Manuscript Number: FOODCHEM-D-13-02492R1

Title: Lupine protein hydrolysates inhibit enzymes involved in the inflammatory pathway

Article Type: Research Article (max 7,500 words)

Keywords: lupine protein hydrolysates; anti-inflammatory activity; bioactive peptides

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Abstract: Lupine protein hydrolysates (LPHs) were obtained from a lupine protein isolate (LPI) by enzymatic hydrolysis using two proteases, Izyme AL and Alcalase 2.4 L, and their potential anti-inflammatory capacities were studied by determining their in vitro inhibition of the following enzymes that are involved in the inflammatory process: phospholipase A2 (PLA2), cyclooxygenase 2 (COX-2), thrombin, and transglutaminase (TG). The strongest inhibitory activities toward PLA2 and TG were found in the hydrolysates obtained by hydrolysis with Izyme and subsequently with Alcalase, with more than 70% inhibition obtained in some cases. All of the hydrolysates tested inhibited more than 60% of the COX-2 activity. In no case did the percentage of thrombin activity inhibition exceed 40%. The best inhibitory activities were found in the LPH obtained after 15 min of hydrolysis with Alcalase and in the LPH obtained after 60 min of hydrolysis with Izyme followed by 15 min of hydrolysis with Alcalase. Enzyme kinetic analyses were conducted to determine the K_m and V_{max} parameters of these two hydrolysates using the Lineweaver-Burk equation. Both hydrolysates competitively inhibited the thrombin and PLA2 activities. In the case of COX-2 and TG, the inhibition appeared to be the mixed type.

HIGHLIGHTS

- Lupine protein isolate was hydrolysed using Alcalase 2.4 L and/or Izyme AL
- Protein hydrolysates inhibited some enzymes involved in the inflammatory pathway
- Lupine protein hydrolysates may act as potential anti-inflammatory agents

LUPINE PROTEIN HYDROLYSATES INHIBIT ENZYMES INVOLVED IN THE INFLAMMATORY PATHWAY

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Abstract

Lupine protein hydrolysates (LPHs) were obtained from a lupine protein isolate (LPI) by enzymatic hydrolysis using two proteases, Izyme AL and Alcalase 2.4 L, and their potential anti-inflammatory capacities were studied by determining their in vitro inhibition of the following enzymes that are involved in the inflammatory process: phospholipase A2 (PLA₂), cyclooxygenase 2 (COX-2), thrombin, and transglutaminase (TG). The strongest inhibitory activities toward PLA₂ and TG were found in the hydrolysates obtained by hydrolysis with Izyme and subsequently with Alcalase, with more than 70% inhibition obtained in some cases. All of the hydrolysates tested inhibited more than 60% of the COX-2 activity. In no case did the percentage of thrombin activity inhibition exceed 40%. The best inhibitory activities were found in the LPH obtained after 15 min of hydrolysis with Alcalase and in the LPH obtained after 60 min of hydrolysis with Izyme followed by 15 min of hydrolysis with Alcalase. Enzyme

26 kinetic analyses were conducted to determine the K_m and V_{max} parameters of these two
27 hydrolysates using the Lineweaver-Burk equation. Both hydrolysates competitively
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31

1. Introduction

Chronic inflammation is involved in the onset and development of numerous chronic diseases, such as cardiovascular diseases, cancer, diabetes, obesity, arthritis, neurological diseases, pulmonary diseases, psychological diseases, and autoimmune diseases (Sung, Prasad, Gupta, Patchva, & Aggarwal, 2012). Inflammation is one of the complex biological responses of the immune system of an organism. In the initial steps, the inflammatory response is mediated by phospholipase A₂ (PLA₂), which is elevated in many inflammatory disorders. PLA₂ hydrolyses the sn-2 ester bond of glycerophospholipids to produce free fatty acids, particularly arachidonic acid (AA), and lysophospholipids (Yedgar, Cohen, & Shoseyov, 2006). AA is metabolised mainly via the cyclo-oxygenase (COX) and lipo-oxygenase enzymatic pathways to produce diverse families of eicosanoids, including prostaglandins (PGs), thromboxanes, and leukotrienes. These eicosanoids are involved in the development of many pathological conditions, particularly inflammation-related processes (Yedgar et al., 2006).

There is a close relationship between inflammation and coagulation. Inflammation-induced coagulation is due to tissue factor, which produces thrombin generation (Levi & van der Poll, 2005). Thrombin is a trypsin-like serine protease that has a central role in haemostasis and thrombosis. Once formed, thrombin cleaves fibrinogen to produce the fibrin mesh of the blood clot (Cirino, Cicala, Bucci, Sorrentino, Maraganore, & Stone, 1996). Thrombin also activates transglutaminase or factor XIII, which cross-links fibrin molecules via the formation of interchain ϵ -(γ -glutamyl)-lysine isopeptide bonds and stabilises the clot by preventing its hydrolysis by proteases (Moreno, 2006).

Diet is thought to be one of the major risk factors for the development of chronic diseases. Thus, modifying the diet could prevent or delay these diseases (Prasad, Sung, & Aggarwal, 2012). Some dietary agents have shown the potential to inactivate

inflammatory molecules by direct binding. Recent studies have provided evidence that peptides can modify crucial regulatory functions of inflammatory processes (Fernandes, Schmidhuber, & Brain, 2009). There are many reports about synthetically derived anti-inflammatory peptides (Rita, Anna, & Susanna, 2013). However, few studies have examined the effect of plant protein hydrolysates on inflammatory markers. It has been reported that soybean protein hydrolysates reduce the level of some of these markers (Vernaza, Dia, Gonzalez de Mejia, & Chang, 2012).

Lupine (*Lupinus angustifolius* L.) is an herbaceous plant typical of the Mediterranean region. Lupine seeds can be incorporated as a protein source in both animal feed and in a variety of human foods. Lupine flour is rich in protein (20-40%), and this content can be increased to more than 90% in protein isolates (Lqari, Vioque, Pedroche, & Millán, 2002). Lupine protein hydrolysates (LPHs) have shown bile-acid binding activity so may have potential applications for reducing cholesterol in hypercholesterolemic patients (Yoshie-Stark & Wäsche, 2004). More recently, lupine proteins have been shown to exert hypotensive and hypotriglyceridemic effects in vivo (Cam & Gonzalez de Mejia, 2012), but there are no reports of their anti-inflammatory activity.

The objective of this work was to determine the effect of LPHs on the activity of four enzymes implicated in the inflammatory process: PLA₂, COX-2, thrombin, and TG. LPHs were obtained by hydrolysis of lupine protein isolate (LPI) with Izyme and Alcalase, two food-grade proteases produced by Novozymes. Izyme has trypsin-like activity whereas Alcalase is a non-specific endoprotease. Both trypsin and Alcalase have been previously used for the generation of bioactive peptides (Korhonen & Pihlanto, 2006). Samples were collected after different periods of incubation, and the activity of the above mentioned enzymes was tested in presence of LPHs with different degrees of

hydrolysis. The LPHs that exhibited more inhibitory activity were selected, and their enzyme kinetic parameters (K_m , V_{max}) were determined.

2. Materials and methods

2.1. Materials

The *L. angustifolius* seeds were provided by Koipesol Semillas, S.A. (Seville, Spain). Izyme AL (564 EU/mL) and Alcalase 2.4 L (2.4 AU/g) were provided by Novozymes (Bagsvaerd, Denmark). PLA₂ from bovine pancreas, albumin (human, essentially fatty acid-free), thrombin from bovine plasma, antithrombin III (AT III), tosyl-gly-pro-arg-*p*-nitroanilide acetate salt (Chromozym), heparin sodium salt, protamine sulfate salt from salmon, TG from guinea pig liver, monodansylcadaverine (dansyl-CAD), dextran-coated charcoal, DL-dithiothreitol (DTT), 2-mercaptoethanol, cystamine dihydrochloride, COX-2 human, AA from porcine liver, N, N, N', N'-tetramethyl *p*-phenylenediamine (TMPD), flunixin meglumine, and hematin (porcine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-palmitoyl-2-pyrenedecanoyl phosphatidylcholine (10-pyrene-PC) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Bovine milk casein was obtained from Calbiochem (Merck Group, Darmstadt, Germany). Iodoacetamide was purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Obtaining the lupine protein isolate

The LPI was obtained using the method of Lqari et al. (2002). Briefly, defatted lupine flour was extracted with 0.25% Na₂SO₃ (w/v) at pH 10.5 for 1 h. After centrifuging the extract at 7500 rpm for 15 min, the supernatant was recovered, and the pellet was extracted again. Both of the supernatants were adjusted to the isoelectric point of lupine

proteins (pH 4.3). The resulting precipitate was washed with distilled water adjusted to pH 4.3 and centrifuged to remove residual salts and other non-protein compounds. Finally, the precipitated proteins were lyophilised and stored at room temperature.

2.2.2. Hydrolysis of the lupine protein isolate

Hydrolysis was conducted in a bioreactor under stirring at a controlled pH and temperature. The LPI was suspended in distilled water (10% w/v), and two types of hydrolysis were performed: one with Izyme followed by Alcalase and one using only Alcalase. The following conditions were used.

Hydrolysis with Izyme and Alcalase: First, the LPI was hydrolysed with Izyme for 1 h at pH 10, 50 °C, and E/S = 100 EU/g protein. Then, a second hydrolysis with Alcalase at pH 8, 50° C, and E/S = 0.3 AU/g protein was conducted for 1 h.

Hydrolysis with Alcalase: The LPI was hydrolysed with Alcalase for 1 h at pH 8, 50 °C, and E/S = 0.3 AU/g protein.

Samples were taken at different times, and enzymes were inactivated by heating at 85°C for 15 min. The supernatants obtained from centrifugation at 8000 rpm for 15 min constituted the LPHs. The LPHs obtained using Izyme followed by Alcalase were designated 0I, 15I, 30I, 60I, 60I+15A, 60I+30A, and 60I+60A. The LPHs obtained using Alcalase were designated 0A, 5A, 10A, 15A, 20A, 25A, 30A, and 60A. The number indicates the time of hydrolysis in minutes.

2.2.3. Determination of the degree of hydrolysis (DH)

The DH, defined as the percentage of peptide bonds cleaved, was determined by the TNBS method according to Adler-Nissen (1979). The total number of amino groups was determined in a sample that had been 100% hydrolysed at 110° C for 24 h in 6 N HCl.

2.2.4. Determination of protein and peptide content

The protein concentrations were determined by elemental microanalysis as % nitrogen content $\times 6.25$ using a Leco CHNS-932 analyser (St. Joseph, MI, USA).

2.2.5. *PLA₂ activity assay*

The PLA₂ activity was evaluated in vitro by measuring the fluorescence produced by its hydrolysis of the 10-pyrene-PC substrate (Cunningham, Maciejewski, & Yao, 2006). PLA₂ was dissolved in a solution of 50 mM Tris, 0.1 M NaCl, and 2 mM CaCl₂ with 0.25% fatty acid-free albumin (pH 7.4) added to a final concentration of 0.5 mg/mL. The substrate (supplied in chloroform) was dried under a nitrogen stream, dissolved in absolute ethanol, and stored at -20° C prior to use. A 5 μ M solution of substrate was prepared in the same buffer used for the enzyme but without albumin. The fluorescence at 355 nm excitation/420 nm emission was measured using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Waltham, MA, USA). The reactions were evaluated for 60 min at 25° C. The concentration of the LPHs was 0.6 mg/mL. A negative control without LPHs and a positive control (0.6 mg/mL flunixin meglumine in the well) were used.

2.2.6. *COX 2 activity assay*

The COX-2 activity was determined according to the method described by Somvanshi et al. (2007) with some modifications. An aliquot of 0.35 μ L of commercial COX-2 was incubated with 5 μ L of LPHs (at 70 μ g protein/ μ L), and 95 μ L of 100 mM Tris, 1 mM EDTA, and 2 μ M hematin pH 8.3 for 1 h at room temperature. The reaction was initiated by adding 50 μ L of 1.6 mM arachidonic acid and 50 μ L of 0.8 mM TMPD dissolved in the same buffer but without hematin and preincubated for 30 min at room temperature. A control without LPHs and a positive control (1.24 mg/mL flunixin meglumine in the well) were used. TMPD oxidation was determined spectrophotometrically by the absorbance at 610 nm using a Multiskan Spectrum

microplate reader (Thermo Labsystem). The initial rate of the reaction was measured during the first 21 s, and the percentage inhibition was calculated.

2.2.7. *Thrombin activity assay*

The thrombin activity was determined by following the increase in the absorbance at 405 nm that accompanies hydrolysis of the chromogenic substrate Chromozym (Ialenti et al., 2001). Fifty microlitres of LPH (at 70 µg protein/µL) was preincubated for 1 h with 5 µL of enzyme (0.136 µg/mL) at room temperature. Then, the mixture was resuspended in 1.45 mL of 50 mM Tris and 150 mM NaCl pH 8.3. The reaction was initiated by adding 0.5 mL of 0.3 mM substrate. The absorbance at 405 nm was measured for 5 min in a Du 640 spectrophotometer (Beckman Coulter). Heparin and AT III were used as the positive control. AT III is a glycoprotein that inactivates several enzymes involved in clotting. The affinity of AT III for thrombin is enhanced by the action of heparin. For this reason, 30 µL of AT III (4.04 µg/mL) and 15 µL of heparin (3.74 g/mL) were incubated for 1 h at room temperature. This mixture was suspended in 1.455 mL of assay buffer and preincubated for 1 h with 5 µL of enzyme (0.136 µg/mL) at room temperature. Afterwards, 0.5 mL of 0.3 mM substrate was added, and the absorbance was measured. A negative control lacking LPHs was used.

2.2.8. *Transglutaminase activity assay*

The method described by Wu & Tsai (2006) was used with some modifications. This method is based on the TG-catalysed incorporation of the fluorescent substrate dansyl-CAD into casein and the subsequent separation of the unreacted fluorescent molecules. The lyophilised enzyme (4.1 U/mg protein) was dissolved in water to a final concentration of 1 mg/mL, and 100 µL aliquots were stored in vials at -80° C until use. Three microliters of enzyme was added to 3 µL of each LPH sample (70 µg protein/µL) and 24 µL of 10 mM Tris buffer pH 7.5, 1 mM EDTA, and 0.025% (v/v) 2-

mercaptoethanol in the wells of a fluorescence microplate that was incubated at room temperature for 60 min. Then, 30 μ L of 100 mM Tris pH 9, 10 mM DTT, 20 mM CaCl_2 , 5 mg/mL casein and 100 μ M dansyl-CAD was added and incubated at 37° C for 30 min. The reaction was stopped by adding 60 μ L of 2 mM iodoacetamide (prepared fresh) for a 2 min incubation on ice. Then, 150 μ L of dextran-coated charcoal at 10 mg/mL was added. The microplate was centrifuged in an Allegra X-22 R centrifuge model (Beckman Coulter) at 1000 rpm for 15 min to pellet the unreacted dansyl-CAD. The supernatants (100 μ L) were carefully withdrawn, and the fluorescence intensity was measured in a microplate reader at excitation and emission wavelengths of 340 and 535 nm, respectively. The enzyme activity was expressed as the net fluorescence per well, which reflects the amount of product formed in the reaction. A control without an inhibitor and a positive control with 0.54 mg/mL cystamine dihydrochloride were used.

2.2.9. Determination of the kinetic constants

The Michaelis constants (K_m and V_{max}) in the presence of the most effective LPHs were calculated for each enzyme by performing activity assays with different substrate concentrations and using Lineweaver-Burk equations.

2.2.10. Statistical analysis

The data are expressed as the mean $\pm$ standard deviation of three independent determinations. The statistical analysis was performed with OriginPro 8 SR0 (OriginLab Corporation (Northampton, MA, USA)). The data were analysed using a one-way ANOVA, followed by Holm-Bonferroni tests for multiple comparisons. Differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1. Study of the degree of hydrolysis

LPI was hydrolysed using two food-grade commercial enzymes, Izyme and Alcalase.

Hydrolysis with Izyme resulted in a DH of approximately 9% (Figure 1A); the DH was not increased by increasing the time of hydrolysis or the enzyme/substrate ratio, which may be due, partially, to the lysine-plus-arginine content of the LPI (15%, data not shown). In analysing the results, it was found that Izyme performance was approximately 60%. Because most bioactive peptides known at present contain 2 to 20 amino acids and have molecular masses of less than 6000 Da (Sarmadi & Ismail, 2010), a second hydrolysis using Alcalase was performed after hydrolysis with Izyme. In this way, the DH was rapidly increased up to 30%.

The rate of hydrolysis of the LPI using only Alcalase was rapid during the first 10 min and then remained almost stable for the following 50 min, reaching a maximum DH of 26% (Figure 1B). A high reaction rate has also been observed for the hydrolysis of other plant proteins using Alcalase (Yust, Millán-Linares, Alcaide-Hidalgo, Millán, & Pedroche, 2012).

3.2. Inhibition of enzymes involved in the inflammatory pathway

3.2.1. Inhibition of PLA₂ activity

PLA₂ enzymes are low molecular weight proteins that catalyse hydrolysis at the sn-2 position of membrane phospholipids, releasing unsaturated fatty acids and liberating AA, a precursor of eicosanoids, including prostaglandins and leukotrienes. These enzymes have been implicated in diverse physiological processes, including asthma, inflammation, carcinogenesis, haemostasis, parturition, maintenance of renal function, pain and fever; indeed, these enzymes are detected in the serum and biological fluids of patients with various inflammatory, autoimmune and allergic disorders (Triggiani, Granata, Frattini, & Marone, 2006). In recent investigations, plant proteins have been shown to act as antioxidants, inhibiting lipid peroxidation, and as anti-inflammatory

agents due to their ability to inhibit PLA₂. One example is turmerin, a protein from *Curcuma longa* L. (Chethankumar, 2010).

Hydrolysis of LPI with Izyme for 60 min increased inhibition of PLA₂ activity up to 68% (Figure 2.A). This value is similar to that showed by the positive control, flunixin. A second step of hydrolysis with Alcalase did not increase or decrease inhibition of PLA₂, which may be attributed to the destruction of inhibitory peptides and the generation of new ones throughout the hydrolytic process. Likewise, the hydrolysis with Alcalase produces smaller peptides which may be more easily absorbed from the gastrointestinal tract. Regarding LPHs obtained using Alcalase, only LPH 60A showed higher PLA₂ inhibition than 0A (Figure 2.B). However, the achieved inhibition was lower than the obtained when LPI was hydrolysed with Izyme and Alcalase.

3.2.2. Inhibition of COX-2 activity

COX catalyses the addition of two moles of molecular oxygen to arachidonic acid, forming prostaglandin G₂ and converting G₂ to PGH₂. Hence, COX performs the critical initial reaction that leads to the formation of pro-inflammatory prostaglandins, thromboxanes and prostacyclins. COX exists in two isoforms, COX-1 and COX-2. COX-1 is considered a constitutive enzyme, whereas COX-2 is an inducible enzyme whose expression occurs in inflammatory cell types and tissues (Gierse, Koboldt, Walker, Seibert, & Isakson, 1999). COX-2 mediates prostaglandin synthesis (PGE₂), which in turn promotes cell proliferation and cytokine secretion and suppresses immune surveillance by mechanisms such as the inhibition of Th1 activation and IL-2 secretion and the suppression of dendritic cells via IL-10. The COX-2 pathway was shown to participate in cases of inflammatory diseases, such as Crohn's disease, and in colon carcinoma (Kanterman, Sade-Feldman, & Baniyash, 2012).

It should be noted that untreated lupine proteins highly inhibited COX-2 activity (Figure 3). The differences between the inhibitory activities of 0I and 0A may be due to the effect of the different pH (10 and 8 respectively), which may alter the structure of the proteins. Hydrolysis of the LPI with Izyme followed by Alcalase did not improve its ability to inhibit COX-2, showing all LPHs tested more than 70% of COX-2 inhibition (Figure 3.A). When the LPI was hydrolysed using only Alcalase, the highest inhibitory activity was observed in the hydrolysate obtained after 15 min of treatment (90%) (Figure 3.B). The inhibition values are remarkable when compared with that of the reference inhibitor, flunixin, which inhibited 98% of COX-2 activity at a concentration slightly less than that of the LPHs (1.24 mg/mL versus 1.75 mg/mL). Moreover, the LPHs are peptide mixtures and only some of the peptides may inhibit COX-2; therefore, purification may result in substances with higher inhibitory activities (Li, Zhou, Huang, Sun, & Zeng, 2012).

3.2.3. Inhibition of thrombin activity

Thrombin acts in the coagulation cascade by catalysing the conversion of fibrinogen into fibrin and, consequently, achieving the formation of a blood clot. Furthermore, thrombin has been found to exert a number of pro-inflammatory and mitogenic effects and has been linked to the development of vascular disease (Morris, Winyard, Blake, & Morris, 1994). Specifically, thrombin stimulates the production of proinflammatory cytokines and interleukins, such as TNF- α , IL-1 and IL-6, and the production of monocyte chemotactic protein (MCP-1) by vascular endothelial cells (Strande & Phillips, 2009). Hydrolysing the lupine proteins with Izyme for 30 min increased their thrombin inhibition by up to 36% (Figure 4.A). When the time of hydrolysis, and therefore the DH, was increased, the thrombin inhibitory activity was reduced to the initial values. When the LPI was hydrolysed only with Alcalase, the highest inhibitory

activity was observed after 15 min of treatment. As in the hydrolytic process with Izyme plus Alcalase, there was not a direct correlation between the increase of the DH and the inhibition of thrombin activity (Figure 4.B). Those results may be due to the destruction of the potentially anti-inflammatory peptides generated initially and the production of new ones in such a way that the thrombin inhibition by LPHs did not depend on the DH but on the generation of specific peptides. These results are similar to those regarding the hypocholesterolaemic and antioxidant effects reported by Yust et al. (2012).

3.2.4. Inhibition of TG activity

TG is a calcium-dependent enzyme that catalyses the covalent crosslinking of the γ -carboxamide groups of peptides bound to glutamine residues with the ϵ -amino groups of peptides bound to lysine residues. In addition, the involvement of TG-2 in the pathogenesis of chronic inflammatory diseases, such as liver cirrhosis, lung fibrosis, and rheumatoid arthritis, has been suggested (Kumar & Metha, 2013). Moreno (2006) reported that short peptides termed antinflammins have anti-inflammatory effects due, at least in part, to their ability to inhibit TG-2.

The rates of TG inhibition by the LPHs obtained with Izyme and Alcalase and with Alcalase are shown in Figures 5.A and 5.B, respectively. The LPHs prepared using Izyme had little effect on the activity of TG, but the addition of a second round of hydrolysis using Alcalase led to 70% inhibition of TG. This result was similar to that of positive control, cystamine, a neuroprotectant used in a number of disease models whose therapeutic benefit has been attributed to its inhibition of the TGs and caspase 3 (Jeitner, Delikatny, Ahlqvist, Capper, & Cooper, 2005), except that the concentration of cystamine used was 130 times less than that of the LPHs. The highest TG inhibition achieved by the LPHs obtained with Alcalase was 51%, which was observed in LPH 15A.

3.3. Determination of kinetic parameters

Considering the observed inhibition of the four enzymes under investigation, two LPHs, 60I+15A and 15A, were selected, and kinetics studies were performed to determine their K_m and V_{max} values using the Lineweaver–Burk equation.

3.3.1. Kinetic parameters of PLA₂ inhibition

The kinetic analysis revealed that the K_m values for PLA₂ inhibition were 3.25 μM for LPH 60I+15A and 1.36 μM for LPH 15A when the inverse of the reaction velocity ($\mu\text{mol}/\text{min}$) and the substrate concentration (μM) (from the Lineweaver-Burk equation) were considered (Table 1). Both of the LPHs seemed to competitively inhibit PLA₂ activity because the V_{max} remained almost constant whereas the ratio V_{max}/K_m decreased (in the case of 60+15A). In the case of LPH 15A, the ratio V_{max}/K_m did not decrease, but the K_m value of LPH 15A was larger than that of the control; therefore, its inhibition also appears to be the competitive type.

3.3.2. Kinetic parameters of COX-2 inhibition

COX-2 exhibited a K_m of 5.146 μM in the kinetic assay without an inhibitor. When LPHs 60I+15A or 15A were added to the reaction medium, the K_m value was 17.51 μM or 15.71 μM , respectively (Table 1). Additionally, the V_{max}/K_m ratio was lower in both cases, indicating that the inhibition of COX-2 by the LPHs was not competitive or uncompetitive but a mixed (non-competitive) inhibition.

3.3.3. Kinetic parameters of thrombin inhibition

As shown in Table 1, the K_m of thrombin inhibition were 23.22 μM for LPH 60I+15A and 32.56 μM for LPH 15A when the inverse of the reaction velocity ($\mu\text{mol}/\text{min}$) and the substrate concentration (μM^{-1}) (from the Lineweaver-Burk equation) were considered. Both LPHs seemed to competitively inhibit thrombin because the V_{max} remained almost constant whereas the V_{max}/K_m ratio decreased.

3.3.4. Kinetic parameters of TG inhibition

The kinetic parameters of TG inhibition were estimated using Lineweaver-Burk plots; the K_m value estimated for dansyl-CAD in this study was 12.61 μM , which was similar to the results obtained by Lorand, Lockridge, Campbell, Myhrman, & Bruner-Lorand (1971), who reported $K_m=14 \mu\text{M}$ with α -casein as an acceptor. For the hydrolysates, K_m 13.44 μM was obtained in the case of the hydrolysate 60I+15A and 14.56 μM was obtained for the 15A hydrolysate. Therefore, the inhibition appears to be the mixed type because the $V_{\max}$ and the $V_{\max}/K_m$ ratio decreased.

4. Conclusions

Hydrolysates of a lupine protein isolate obtained using commercial proteases inhibited the activity of some of the enzymes involved in the inflammatory pathway. Chronic inflammation is associated with the development of several chronic diseases. Therefore, the results of this study indicate the potential use of lupine protein hydrolysates in the production of functional foods. Additionally, the addition of lupine protein hydrolysates as ingredients may improve the nutritional value of a product. However, further studies should be performed to elucidate the anti-inflammatory effects of lupine hydrolysates in vivo.

Acknowledgements

This work was supported by the grant AGL2012-40247-C02-01 from the Spanish Ministry of Economy and Competitiveness.

References

353 Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein
354 hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food*
355 *Chemistry*, 27, 1256–1262.

356 Cam, A., & Gonzalez de Mejia, E. (2012). Role of dietary proteins and peptides in
357 cardiovascular disease. *Molecular Nutrition & Food Research*, 56, 53–66.

358 Chethankumar, M. (2010). Turmerin, a protein from *Curcuma longa* L. prevent
359 oxidative organ damage against *Naja naja* venom phospholipase A2 in experimental
360 animal. *Journal of Current Pharmaceutical Research*, 3, 29-34.

361 Cirino, G., Cicala, C., Bucci, M. R., Sorrentino, L., Maraganore, J. M., & Stone, S. R.
362 (1996). Thrombin functions as an inflammatory mediator through activation of its
363 receptor. *Journal of Experimental Medicine*, 183, 821–827.

364 Cunningham, T. J., Maciejewski, J., & Yao, L. (2006). Inhibition of secreted
365 phospholipase A2 by neuron survival and anti-inflammatory peptide CHEC-9. *Journal*
366 *of Neuroinflammation*, 3, 25. doi: 10.1186/1742-2094-3-25

367 Fernandes, E., Schmidhuber, S. M., & Brain, S. D. (2009). Sensory-nerve-derived
368 neuropeptides: Possible therapeutic targets. *Handbook of Experimental Pharmacology*,
369 194, 393-416.

370 Gierse, J. K., Koboldt, C. M., Walker, M. C., Seibert, K., & Isakson, P. C. (1999).
371 Kinetic basis for selective inhibition of cyclo-oxygenases. *Biochemical Journal*, 339,
372 607- 614.

373 Ialenti, A., Santagada, V., Caliendo, G., Severino, B., Fiorino, F., Maffia, P., et al.
374 (2001). Synthesis of novel anti-inflammatory peptides derived from the amino-acid
375 sequence of the bioactive protein SV-IV. *European Journal of Biochemistry*, 268, 3399-
376 3406.

377 Jeitner, T. M., Delikatny, E. J., Ahlqvist, J., Capper, H., & Cooper, A. J. L. (2005).
378 Mechanism for the inhibition of transglutaminase 2 by cystamine. *Biochemical*
379 *Pharmacology*, 69, 961–970.

380 Kanterman, J., Sade-Feldman, M., & Baniyash, M. (2012). New insights into chronic
381 inflammation-induced immunosuppression. *Seminars in Cancer Biology*, 22, 307-318.

382 Korhonen, H., & Pihlanto, A. (2006) Bioactive peptides: Production and functionality.
383 *International Dairy Journal*, 16, 945-960.

384 Kumar, S., & Mehta, K. (2013) Tissue transglutaminase, inflammation, and cancer: how
385 intimate is the relationship? *Amino acids*, 44, 81-88.

386 Levi, M., & van der Poll, T. (2005). Two-way interactions between inflammation and
387 coagulation. *Trends in Cardiovascular Medicine*, 15, 254–259.

388 Li, Y., Zhou, J., Huang, K., Sun, Y., & Zeng, X. 2012. Purification of a novel
389 angiotensin I-converting enzyme (ACE) inhibitory peptide with an antihypertensive
390 effect from loach (*Misgurnus anguillicaudatus*). *Journal of Agricultural and Food*
391 *Chemistry*, 60, 1320–1325.

392 Lorand, L., Lockridge, O. M, Campbell, L. K, Myhrman, R., & Bruner-Lorand, J.
393 (1971). A continuous fluorescent method suited for automating measurements of factor
394 XIII in plasma. *Analytical Biochemistry*, 44, 221-231.

395 Lqari, H., Vioque, J., Pedroche, J., & Millán, F. (2002). *Lupinus angustifolius* protein
396 isolates: chemical composition, functional properties and protein characterization. *Food*
397 *Chemistry*, 76, 349-356.

398 Moreno, J. J. (2006). Effects of antiinflammins on transglutaminase and phospholipase A₂
399 activation by transglutaminase. *International Immunopharmacology*, 6, 300–303.

400 Morris, R., Winyard, P. G., Blake, D. R., & Morris, C. J. (1994). Thrombin in
401 inflammation and healing: relevance to rheumatoid arthritis. *Annals of the Rheumatic*
402 *Diseases*, 53, 72-79.

403 Prasad, S., Sung, B., & Aggarwal, B. B. (2012). Age-associated chronic diseases require
404 age-old medicine: Role of chronic inflammation. *Preventive Medicine*, 54, S29-S37.

405 Selvatici, R., Siniscalchi, A., & Spisani, S. (2013). Effects of synthetic peptides on the
406 inflammatory response and their therapeutic potential. *Mini Reviews in Medicinal*
407 *Chemistry*, 13, 553-564.

408 Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A
409 review. *Peptides*, 31, 1949-1956.

410 Somvanshi, R. K., Kumar, A., Kant, S., Gupta, D., Singh, S. B., Das, U., et al. (2007).
411 Surface plasmon resonance studies and biochemical evaluation of a potent peptide
412 inhibitor against cyclooxygenase-2 as an anti-inflammatory agent. *Biochemical and*
413 *Biophysical Research Communications*, 361, 37-42.

414 Strande, J. L., & Phillips, S. A. (2009). Thrombin increases inflammatory cytokine and
415 angiogenic growth factor secretion in human adipose cells *in vitro*. *Journal of*
416 *Inflammation*, 6, 4, doi:10.1186/1476-9255-6-4.

417 Sung, B., Prasad, S., Gupta, S. C., Patchva, S., & Aggarwal, B. B. (2012). Chapter 3 –
418 Regulation of inflammation-mediated chronic diseases by botanicals. *Advances in*
419 *Botanical Research*, 62, 57-132.

420 Triggiani, M., Granata, F., Frattini, A., & Marone, G. (2006). Activation of human
421 inflammatory cells by secreted phospholipases A2. *Biochimica et Biophysica Acta*,
422 1761, 1289-1300.

423 Vernaza, M. G., Dia, V. P., González de Mejía, E., Chang, Y. K. (2012). Antioxidant
424 and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours.
425 *Food Chemistry*, 134, 2217–2225.

426 Wu, Y. W., & Tsai Y. H. (2006). A rapid transglutaminase assay for high-throughput
427 screening applications. *Journal of Biomolecular Screening*, 11, 836-843.

428 Yedgar, S., Cohen, Y., Shoseyov, D. Control of phospholipase A2 activities for the
429 treatment of inflammatory conditions. (2006). *Biochimica et Biophysica Acta*, 1761,
430 1373–1382.

431 Yoshie-Stark, Y., & Wäsche, A. (2004). In vitro binding of bile acids by lupin protein
432 isolates and their hydrolysates. *Food Chemistry*, 88, 179-184.

433 Yust, M. M., Millán-Linares, M. C., Alcaide-Hidalgo, J. M., Millán, F., & Pedroche, J.
434 (2012). Hypocholesterolaemic and antioxidant activities of chickpea (*Cicer arietinum*
435 L.) protein hydrolysates. *Journal of the Science of Food and Agriculture*, 92, 1994-
436 2001.

437

438 **FIGURE CAPTIONS**

439 **Figure 1.** Time-course of hydrolysis of LPI by Izyme and Alcalase (A) and Alcalase
440 (B).

441 **Figure 2.** Inhibition of PLA2 activity by LPHs obtained using Izyme followed by
442 Alcalase (A) and using Alcalase (B). Values marked with different letter are
443 significantly different ($P<0.05$).

444 **Figure 3.** Inhibition of COX-2 activity by LPHs obtained using Izyme followed by
445 Alcalase (A) and using Alcalase (B). Values marked with different letter are
446 significantly different ($P<0.05$).

447 **Figure 4.** Inhibition of thrombin activity by LPHs obtained using Izyme followed by
448 Alcalase (A) and using Alcalase (B). Values marked with different letter are
449 significantly different ($P<0.05$)

450 **Figure 5.** Inhibition of transglutaminase activity by LPHs obtained using Izyme
451 followed by Alcalase (A) and using Alcalase (B). Values marked with different letter
452 are significantly different ($P<0.05$).

TABLES

Table 1. Values of $V_{\max}$ and the ratios $V_{\max}/K_m$ for controls and inhibitors obtained from Lineweaver-Burk equation for different enzymes.

ENZYME	PLA2		COX-2		Thrombin		TG-2	
	$V_{\max}$	$V_{\max}/K_m$	$V_{\max}$	$V_{\max}/K_m$	$V_{\max}$	$V_{\max}/K_m$	$V_{\max}$	$V_{\max}/K_m$
Control	1.01	0.83	15.64	3.04	17.88	1.09	7288	578.0
60I+15A	1.16	0.36	11.66	0.67	17.50	0.75	5072	377.4
15A	0.97	0.71	11.06	0.70	19.27	0.59	5871	403.2

$V_{\max}$ is expressed in $\mu\text{mol}/\text{min}$

K_m is expressed in μM

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